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Abstract: This study reports on the structure of the antennal lobe of the pigeon louse, Columbicola columbae. Anterograde staining of antennal receptor neurons revealed an antennal lobe with a few diffuse compartments, an organization distinct from the typical spheroidal glomerular structure found in the olfactory bulb of vertebrates and the antennal lobe of many other insects. This anatomical arrangement of neuronal input is somewhat reminiscent of the aglomerular antennal lobe previously reported in psyllids and aphids. As in psyllids, reports on the odor-mediated behavior of C. columbae suggest that the olfactory sense is important in these animals and indicates that a glomerular organization of the antennal lobe may not be necessary to subtend odor-mediated behaviors in all insects. The diffuse or aglomerular antennal lobe organization found in these two Paraneopteran insect orders might represent an independently evolved reduction due to similar ecological constraints.

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Highlights:

- We studied the structure of the antennal lobe of the pigeon louse.
- We stained antennal receptor neurons.
- Lice presented a diffuse compartmentalized organization of the antennal lobes.
- Findings challenge the notion that primary olfactory brain centers are always organized in glomeruli.

1. Introduction

The primary olfactory brain centers of many vertebrates and insects exhibit a distinctive anatomy that is readily recognized by the organization of the neuropil into globular units called olfactory glomeruli (Hildebrand and Shepherd, 1997). In many insects, these glomeruli comprise the structural and functional units of the antennal lobes (ALs), the first-order olfactory brain areas, which receive receptor neuron input from peripheral sensory sensilla. The number, size, and spatial arrangement of AL glomeruli are species-specific and consistent among different individuals of the same species (Anton and Homberg, 1999). The number of glomeruli found in the ALs of insects ranges from 10 to 1000 (Rospars, 1988). Since olfactory sensory neurons expressing the same receptor protein converge on a single glomerulus (Gao et al., 2000), the number of glomeruli approximately reflects the spectrum of expressed receptor genes. Furthermore, glomeruluar size appears to be correlated to the number of incoming afferents of a particular type (Anton and Homberg, 1999). This is evidenced in the sexually dimorphic ALs, associated with mate finding, that have been described in several Hymenopteran, Lepidopteran, and Dictyopteran species (Rospars, 1988). In these orders a macroglomerular complex, i.e. a male specific glomerular aggregation that is involved in the processing of sex pheromone input, has been reported and its units found to be larger than ordinary glomeruli (e.g. Vickers and Christensen, 2003). This glomerular characteristic stems from the large number of sex-pheromone olfactory receptor neurons (ORNs) on the antenna which confer a high sensitivity to the female produced sex pheromone. The functional significance of glomeruli is supported by a wide range of studies in a variety of insect species (e.g. Rodrigues, 1988; Hildebrand,

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1996; Galizia et al., 1999). Since each physiological type of ORN projects into a specific glomerulus, they form the basis of a so-called chemotopic map in the AL (Vosshall et al., 2000) in which qualitative features of differing odor mixtures are represented by unique combinations of spatial activity.

In this study, we investigated the AL morphology of the slender pigeon louse *Columbicola columbae* (Phthiraptera: Ischnocera), an ectoparasite of the Rock Pigeon, *Columba livia*. The antennae of this insect consists of five annuli (scape, pedicel, and three flagellomeres) but only the last two flagellomeres bear sensilla other than mechanoreceptors (Smith, 2001). In spite of the fact that *C. columbae* harbors few sensilla on its antennae, behavioral reports have shown that this insect is attracted to the smell of its host (Rakshpal, 1959) and to that of the hippoboscid fly *Pseudolynchia canariensis*, involved in the phoretic behavior of this species of lice (Harbison et al., 2009; Harbison and Clayton, 2011). Our investigations of *C. columbae* ALs revealed a non-globular compartmentalization of the neuropil reminiscent of the aglomerular AL found in psyllids and aphids (Kristoffersen et al. 2008; Kollmann et al. 2011). The lack of defined glomerular structures in the ALs of *C. columbae*, as well as in that of psyllids and aphids, suggests that a glomerular configuration is not always a hallmark feature of insect antennal lobes.

2. Materials and Methods

2.1 Insects

C. columbae females and males were obtained from Dr. Dale H. Clayton, University of Utah.

2.2 Brain autofluorescence

Individual brains of male and female lice were dissected in saline solution under a microscope and then fixed with 2.5% formaldehyde in 0.1 M phosphate buffered saline solution (PBS) overnight. Brains were then removed from the fixative, placed in 2% glutaraldehyde for 24 hours, and observed using a 1µm thickness of optical sections with a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Inc., Thornwood, NJ).

2.3 Antennal backfills

Live individual lice were placed on a Petri dish (35x10 mm polystyrene, BD Falcon®) and restrained on double-sided sticky tape (3M Scotch®). Either the right or left antenna was excised below the first flagellomere to ensure that the receptors' projections of all non-tactile sensilla (Smith, 2001) could be stained. A glass electrode filled with cobalt-lysine (2.38 g cobaltous chloride plus 5 g L-lysine in 20 ml of distilled water, lowered to a pH of 7.2-7.4 by HCl) or dextran tetramethylrhodamine (3% in distilled water, 3,000 MW, lysine-fixable; Molecular Probes, Eugene, OR) solution was slid over the cut-tip of the antenna and left for 4-5 hours at 4°C. A moistened piece of cotton maintained a high relative humidity in the sealed Petri dish. Insects were then fixed with 2.5% formaldehyde in 0.1 M PBS overnight at 4°C. Those specimens stained with dextran rhodamine (N=11) where then dehydrated in an ethanol series, cleared with methyl salicylate and examined with a laser scanning confocal microscope (Zeiss LSM 510). Those specimens stained with cobalt-lysine (N=4) where subsequently subjected to silver intensification (Bacon and Altman, 1977), dehydrated through a graded series of ethanol, placed in methyl salicylate, and examined as whole mounts under a light microscope. Whole insects were embedded in Durcupan resin (Electron Microscopy Sciences, Ft. Washington, PA), sectioned at 1 μm and mounted on microscope slides. Sections were counterstained using modified Lee's methylene blue-basic fuchsin solution (Lee et al., 2006) and examined at 40-100 X. Digital images were taken with a charge-coupled device (CCD) camera (Carl Zeiss AxioCam HRc).

2.4 Data analysis

Zeiss LSM confocal images were imported into ImageJ (http://rsb.info.nih.gov/ij/) and the volumes of ALs, optic lobes (OLs), and entire brains calculated. Male and female comparisons were performed by means of a Chi-square test of independence. Volumes of the OLs and thus, those of the whole brains, do not include the first neuropil region (i.e. the lamina).

3. Results

There are few sensory structures on the antennae of *C. columbae* some of which exhibit morphological features consistent with an olfactory function (e.g. sensilla placodea and sensilla coeloconica; Smith, 2001). The small number of olfactory sensilla present on the antennae and the fact that these insects are permanent ectoparasites of birds is

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consonant with a minor role for olfaction in these insects. However, until now, no description of the primary olfactory processing center in the brain, i.e. the AL, of this or any other species of lice has been reported. By using two different methods for anterograde staining of antennal sensory neurons, we have been able to visualize the structure of the AL of this louse (Figure 1, 2). Our analysis of *C. columbae* ALs reveals an atypical organization of this structure in contrast to the usual glomerular compartmentalization seen in most other insects that have been examined to date (Rospars, 1988; Anton and Homberg, 1999). Figure 1 shows the localization of the AL in the brain and the atypical organization of this brain region. The AL neuropil (as seen in semi-ultrathin sections; data not shown) was similar to that of other brain areas that typically never exhibit a glomerular arrangement such as the Central Body. Even though the antennal lobe neuropil appears to exhibit heterogeneity in staining (Figure 2), this demarcation is very different from the spheroidal glomeruli that have been reported in most other insects and vertebrates and more likely reflects accretions of synaptic contacts similar to those detailed in psyllids and aphids (Kristoffersen et al., 2008). A 3D reconstruction of the AL with ORNs stained anterogradely by rhodamine dextran, and what appears to be the antennal mechanosensory and motor complex (AMMC; Figure 2), further supports the conclusion of a weakly compartmentalized AL.

Since no clearly defined glomeruli were identified in the AL of *C. columbae*, it is not possible to unequivocally conclude whether a sexually dimorphic region of the AL exists (as seen for example in moths, Rospars and Hildebrand, 2000). However, our results show that the AL of both males and females (female data not shown) have no gross morphological differences with either of the two staining techniques used (i.e. cobalt-lysine and rhodamine dextran staining). In both sexes the ALs are relatively small cloud-shaped structures, measuring around 35µm in diameter. Receptor neurons from the antenna appear to terminate either in the AL or the AMMC (Figure 2), indicating that no taste sensilla are found on the antenna (corroborated by the sensilla described in Smith, 2001). The AL volumes of females (14440±163 μm^3, SE, n=4) and males (14103±239 μm^3, SE, n=7) showed no significant difference (*P*=0.27) further supporting the notion that a sexually dimorphic region in the AL is absent. Furthermore, the ALs make up about 2.5% of the total brain volume of *C. columbae*, a small percentage compared to other insects (e.g. 9% in ants; Gronenberg et al., 1996). Still, the olfactory neuropil is more developed than that allocated to vision. Due to their ectoparasitic lifestyle, lice have vestigial eyes that are connected to the optic lobes by very thin optic nerves. Both the medulla and lobula have a combined volume of 1860±21μm^3 (SE; n=4) in females and 1785±39μm^3 (SE; n=7) in males making up around 0.3% of the total brain volume.

Discussion

Both lice (Phthiraptera) and psyllids (Hemiptera: Homoptera) are classified as Paraneopteran orders (Grimaldi and Engel, 2005). Thus, if an aglomerular or diffuse compartmentalization of the AL neuropil is an ancestral trait for this group, it might also be present in other Paraneopteran orders such as the Psocoptera and the Thysanoptera. In fact, the only study on the morphology of the ALs of book lice (Psocoptera) reported that glomeruli cannot be distinguished (Stöwe, 1943 *cited in*

Schachtner et al., 2005). Besides these insect orders, an aglomerular appearance of the brain area receiving olfactory input has only been reported in anosmic insects (primarily Ephemeroptera and Odonata; e.g. reviewed in Schachtner et al., 2005; Strausfeld et al., 2009; Crespo, 2011). Comparisons of the structure of the *C. columbae* AL with that of related taxa exhibiting varying degrees of parasitism could help to elucidate whether this morphological feature is an evolved reduction associated with a highly parasitic lifestyle or a more deeply embedded trait within this group. A recent sequencing study of the human body louse (*Pediculus humanus humanus*), an obligate parasite of humans, revealed a reduced genome that was deficient in genes that encode for proteins associated with sensory functions (chemosensory and visual) (Kirkness et al., 2010). Only 10 odorant receptor genes were identified and these data suggest that a parasitic life history leads to a loss of genes associated with detection of environmental cues in general (Kirkness et al., 2010). It seems reasonable to speculate that this reduction in odorant receptor genes would lead to a commensurate reduction in the number of antennal lobe compartments in the human body louse.

The morphological similarity between the ALs of parasitic *C. columbae* and free-living psyllids could be the result of convergent evolution due to certain characteristics of the environment that these two groups of insects inhabit. Limited need for and use of olfactory cues could lead to a reduction in the number of sensory afferents from the antennae accompanied by a commensurate reduction in the number of AL compartments and other structural changes. An aglomerular AL organization was previously reported in the carrot psyllid *Trioza apicalis* (Hemiptera: Homoptera) in spite of this insect's dependency on olfactory cues to find hosts and migrate to shelter plants during seasonal changes (Kristoffersen et al., 2008). Thus, the few olfactory sensilla present on the antennae and the diffuse structure of the AL neuropil in *C. columbae*, as in *T. apicalis*, may not indicate that olfaction plays a minor role in this insects' life history. In fact, *C. columbae* has been shown to be attracted to the smell of pigeon feathers and other host related odors (Rakshpal, 1959), as well as to olfactory cues originating from the hippoboscid fly *P. canariensis*, which is involved in the phoretic movements of this species of lice (Harbison et al., 2009). Kristoffersen et al. (2008) proposed two explanations for the reduced number of ORNs found in *T. apicalis* which in turn might explain the aglomerular structure of the AL in that species: (1) as an adaptation to prevent desiccation during the winter, and (2) due to the strong smell that this psyllid's hosts emanate and their occurrence in large stands. These two explanations hold true for lice as well. First, lice are known to do poorly at low humidity since they acquire moisture by absorbing it from the surrounding air. At low relative humidity, these insects are unable to maintain their water balance (Rudolph, 1983). So, a reduction in the number of olfactory sensilla of lice might also be explained by this environmental constraint. Second, as permanent ectoparasites of birds, lice are exposed to the abundant and constant odor of their hosts which might lessen the need for sensitive host detection abilities. Nonetheless, evidence suggests that these animals are attracted by host odor and that of hippoboscid flies which they use to support their phoretic lifestyle. However, little is known about odor-mediated communication within and between different lice species. Such information would be necessary to facilitate studies of the physiological properties of the AL compartments in

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C. columbae and whether they bear any functional resemblance to those of typical olfactory glomeruli in other insect taxa.

This study provides the first detailed report on the primary olfactory centers of insects belonging to the Order Phthiraptera. Our results show that the structure of the *C. columbae* AL exhibited weakly defined compartments without clearly delineated spheroidal glomeruli, a condition similar to that previously reported in the psyllid, *T. apicalis* (Kristoffersen et al., 2008). Even though both homopterans and phthirapterans share a common ancestor, the presence of this trait might be the result of convergent evolution due to similarities in their natural environment.

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Figure Legends

Figure 1. Morphological structure of the antennal lobe (AL) of the male louse *Columbicola columbae*. Dorsal view of brain with anterograde stains from the antenna with cobalt-lysine. Black arrows: lateral head cuticle removed; white arrow: AL stained with cobalt-lysine. Cobalt-lysine staining throughout the AL is heterogeneous, most likely reflecting areas with a greater concentration of synaptic contacts.

Figure 2. Projection of series of confocal images show terminals of antennal nerve axons in the antennal lobe (AL). Axons were stained anterogradely with rhodamine dextran in male lice. Staining shows olfactory neurons targeting the right (and left, in the inset figure) AL and probably mechanosensory neurons targeting the antennal mechanosensory and motor complex (AMMC; white arrow). Inset figure shows a more detailed view of the structure of the AL in a different specimen. Heterogeneous staining of the AL is consistent with that observed with cobalt-lysine staining. The neuropil appears to exhibit three weakly delineated compartments (although a few more could also be discerned in the preparation) but neither of the staining techniques utilized in this study revealed a glomerular architecture typical of that observed in many other insects.

Figure 1
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Figure 2
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